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Review 综 述

萜类合成酶定向进化的新思路

胡智慧，翁彦如，谌柄旭，于爱群，肖冬光*

天津科技大学生物工程学院，教育部工业发酵微生物重点实验室，微生物代谢与发酵过程控制技术工程中心，天津 300457

摘要：萜类化合物是天然产物中种类最多且主要存在于植物和微生物体内的一类化合物。随着越来越多具有应用价值的萜类化合物被挖掘，其应用前景引起了人们的关注，但由于含量低、提取成本高等缺点，因此制约了萜类化合物的广泛应用。合成生物学的兴起，为异源合成具有应用价值的萜类化合物提供了新思路，使构建定向、高效的微生物细胞工厂成为现实。萜类合成酶常作为萜类化合物异源合成代谢调控的靶酶，但天然的萜类合成酶存在催化效率低、底物专一性差、立体/区域选择性差、稳定性差等问题，严重影响萜类化合物的产量。萜类合成酶的定向进化可以有效地解决上述问题，为实现微生物细胞工厂异源、高效合成萜类化合物奠定基础。本文综述了近年来酶的定向进化技术的最新进展及应用，并提出了萜类合成酶定向进化的策略。

关键词：萜类化合物，萜类合成酶，合成生物学，定向进化

萜类化合物是种类最多的一类天然产物，具有抗癌、抗过敏等多种生物活性及功能，在食品、日化、医疗等领域受到了广泛关注，展现了巨大的应用潜力和广阔的市场前景[1]。根据其所含异戊二烯数目的不同可以分为单萜(C_{10})、倍半萜(C_{15})、二萜(C_{20})、三萜(C_{30})、四萜(C_{40})和多萜等[2]。

近年来，随着合成生物技术的兴起，为微生物异源合成天然活性化合物带来了全新的理念与工具，打破了物种间的界限，使微生物异源合成萜类化合物成为现实。构建定向、高效的异源合成萜类化合物的微生物细胞工厂，实现微生物发酵法替换传统的植物提取法，具有重要的经济与社会效益[3]。

萜类合成酶是萜类化合物高效异源合成的瓶颈，主要存在催化效率低、底物专一性差、立体/区域选择性差、稳定性差等问题。为了解决上述

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*通信作者。Tel：+86-22-60600019；E-mail：xiao99@tust.edu.cn

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问题，可采取萜类合成酶体外定向进化的策略，组合优化萜类合成酶的多项参数，进而提高酶的整体性能。酶的传统定向进化技术，如易错PCR(Error-prone PCR)、DNA混组(DNA shuffling)、序列饱和突变(Sequence saturation mutagenesis SeSaM)、随机引发体外重组(Random-priming recombination)等，存在突变效率低、筛选工作量大等缺点，制约了酶分子体外定向进化的应用。近年来开发出了一系列基于组合活性中心饱和突变(Combinatorial active-site saturation test，CAST)及迭代饱和突变 (Iterative saturation mutagenesis，ISM)的半理性设计的新方法，包括单密码子饱和突变 (Single code saturation mutagenesis，SCSM)、双密码子饱和突变(Double code saturation mutagenesis，DCSM)和三密码子饱和突变 (Triple code saturation mutagenesis，TCSM)。通过构建“小而精”的高质量突变体文库，对特定靶点进行组合突变，最终获得性能改进或具有新功能的酶，极大地拓宽了酶的应用范围[4]。

本文主要针对萜类合成酶的定向进化提出一些新思路：在已知或未知萜类合成酶的结构信息及催化机制的情况下，通过在线软件(SWISS-MODEL、Phyre 2)预测未解析萜类合成酶的三级结构，与相应已解析的萜类合成酶比对，找出其活性口袋或者具有催化活性的位点，选择合适的突变策略，构建“小而精”的突变文库[4]，从中筛选出高活性的萜类合成酶，为后续萜类化合物异源合成奠定基础。本文还对萜类合成酶今后的应用及发展前景进行了展望。

1 萜类合成酶的结构及功能

萜类合成酶是萜类化合物生物合成中的一类关键酶，包括单萜合成酶、倍半萜合成酶、二萜合成酶等。Christianson[5]提出根据起始碳正离子形成的方式，可将萜类合成酶分为三类(图 1)：Class Ⅰ，主要包括单萜、倍半萜以及二萜合成酶，其通过金属离子(Mg^{2+}、Mn^{2+})的离子化作用脱去底物的焦磷酸基团；Class Ⅱ，主要包括部分二萜合成酶、三萜合成酶等，其通过天冬氨酸侧链形成的碳碳双键的质子化作用脱去底物的焦磷酸基团；Class Ⅰ和Class Ⅱ的组合体。Christianson还指出萜类合成酶有3个不同的蛋白结构域(α，β，γ)，同一种萜类合成酶可由不同的结构域组合而成(图 2)。Oldfield等[6]也指出Class Ⅰ的催化结构域是天冬氨酸富集区(DDXXD/$[Mg^{2+}]_3$)，主要是通过离子化作用脱去底物的焦磷酸基团；Class Ⅱ的催化结构域也是天冬氨酸富集区(DXDD)，但其催化机制与Class Ⅰ不同，主要是通过质子化作用脱去底物的焦磷酸基团。Class Ⅰ和Class Ⅱ组合体既能通过离子化作用脱去底物的焦磷酸基团，也能通过质子化作用脱去底物的焦磷酸基团。不同的萜类合成酶决定了萜类碳骨架的多样性，也决定了其功能的多样性(表 1)。

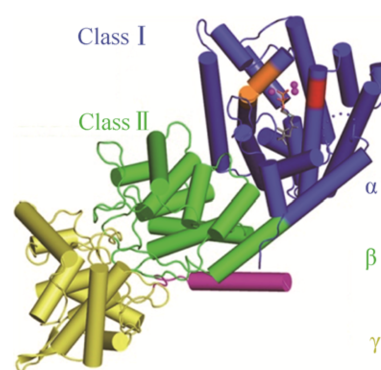

图 1. 萜类合成酶的类别(Class Ⅰ，Class Ⅱ，Class Ⅰ and Class Ⅱ)及结构域[5](α，β and γ)[5]

Figure 1. The main classes (Class Ⅰ, Class Ⅱ, Class Ⅰ and Class Ⅱ) and domains (α, β and γ) of terpenoid synthases[5].

表 1. 不同萜类化合物的功能与应用

Table 1. The functions and applications of different terpenoids

Applications	Terpenoids	Functions	References
Medical field	Paclitaxel	Treatment of ovarian cancer, breast cancer etc.	[7]
	Cucurbitacin E	Treatment of breast cancer, liver cancer etc.	[8]
	Triterpenoid saponin	Anti-inflammation, anti-allergy, anti-virus, treatment of leukemia, blood sugar etc.	[9]
	Ginsenoside CK	Anti-phlogistic, anti-cancer etc.	[10]
	β-carotene	Anti-oxidant, anti-cancer etc.	[11]
Perfume cosmetics	Perilla alcohol	Food flavour	[12]
	Linalool	Essential oil	[13]
	Menthol	Food perfumer	[14]
	Limonene	Essential oil , food perfumer and anti-cancer	[15]
Fuel substitute	Farnesene	Biofuel precursor	[16]
	Bisabolene	Advanced biofuel precursor	[17]

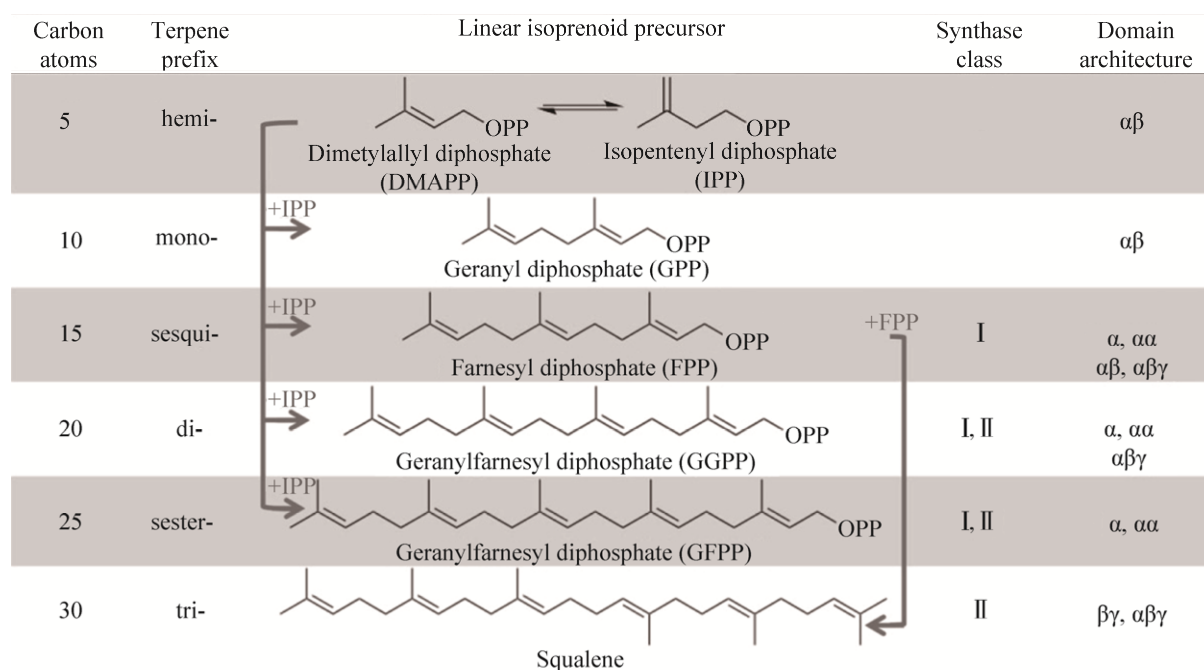

图 2. 萜类合成酶的分类、结构域组成、催化底物及相应的产物[5]

Figure 2. Classification of terpenoid synthase, structure domain composition, catalytic substrates and corresponding products[5].

2 酶定向进化的策略

根据突变体文库的构建方法，可将酶的定向进化分为非理性设计、半理性设计和理性设计 3 种策略(表 2)。其大致思路是通过实验室条件下模拟酶的自然进化，对目的基因进行重复多轮的突变、表达和筛选，从而在短时间内完成自然界中需要成千上万年的进化，最终获得性能改进或具有新功能的酶[18]。

表 2. 酶定向进化的不同策略

Table 2. Different strategies for directed evolution of enzymes

Classifications	Strageties	Requirements	Applications	References
Non-rational design	Site-specific mutagenesis	No protein sequences, structure-function relationships	Identification a key active site residue (Tyr to Val) that influences the stereochemistry of enoylreduction	[19]
	Saturation mutagenesis	No protein sequences, structure-function relationships	Enhancing the enantioselective mutants of the thermally robust phenyl acetone monooxygenase (PAMO)	[20]
	epPCR	No protein sequences, structure-function relationships	Enhancing the enantioselectivity of an epoxide hydrolase	[21]
	DNA shuffling	No protein sequences, structure-function relationships	Generating highly recombined genes and evolved enzymes	[22]
	SeSam	No protein sequences, structure-function relationships	A novel method for directed evolution that truly randomizes a target sequence at every single nucleotide position	[23]
Rational design	Computer-assisted rational design	Systematically analyzing the codependencies between the lengths andpacking geometry of successive secondary structure elements and the backbone torsion angles of the loop linking them	Providing the foundation for custom design of protein structures performing desired functions	[24]
Semi-rational design	REAP	Phylogenetic analysis	Engineering polymerases to accept dNTP-ONH2	[25]
	ProSAR	Sequence-activity data set	Improving the productivity of a halohydrin dehalogenase	[26]
	KnowVolution	Structural model	Reducing oxygen dependency and increasing specific activity of a glucose oxidase	[27–28]
	SCSM	Structural model	Enhancing or inverting the stereoselectivity of enzymes for use in organic chemistry or biotechnology	[29–30]
	DCSM	Structural model	Exploring the efficacy of double code saturation mutagenesis (DCSM) in which the reduced amino acid alphabet comprises	[31]
	TCSM	Structural model	Efficient tuning of the stereoselectivity of an epoxide hydrolase	[32–33]

REAP: Reconstructed evolutionary adaptive path, ProSAR: Protein sequence activity relationship analysis. Based strategy. KnowVolution: Knowledge gaining directed eVolution; SeSaM: Sequence saturation mutagenesis; SCSM: single code saturation matugenesis; DCSM: double code saturation matugenesis; TCSM: triple code saturation matugenesis.

3 萜类合成酶定向进化的实例

近年来国内外科研工作者以酿酒酵母、大肠杆菌、解脂耶氏酵母、蓝藻等作为底盘微生物，已成功实现萜类化合物的异源合成，但萜类合成酶一直是限制萜类化合物异源、高效合成的关键酶。针对天然的萜类合成酶存在的问题，研究者已采取不同的定向进化策略如易错 PCR、定点突变、饱和突变等，对萜类合成酶的催化结构域、活性位点进行挖掘，改造原有酶的参数，进而改善酶的催化性能，实现萜类化合物在微生物细胞中定向、高效地异源合成。

Nigel S. Scrutton 课题组通过对植物中的单萜环化酶/合成酶(mTC/Ss)的序列进行多重比对，挖掘出影响单萜环化酶/合成酶催化活性的 3 个相对保守的区域(表 3)，再结合定点突变、合成生物学、

分子动力学模拟、QM/MM 等策略，对保守区域进行定向进化，其中 LimS region 2 (S454G，C457V，M458I) 对柠檬烯的产量有显著提升；PinS region 1 (C373I，H374A，I375L)、PinS region 2 (S481I，H483G，R484P，S486I) 对蒎烯合成酶的催化活性有显著影响；FenS region 1 (T344I)、FenS region 2 (T450G，C451G，T453V)对茴香醇合成酶酶活影响严重，突变体中未检测到茴香醇，这些结果为单萜环化酶/合成酶理性设计奠定了基础，同时也进一步阐明保守序列结构与功能之间的关联[34]。

Daisuke Umeno 课题组首先利用易错 PCR 的策略对蒎烯合成酶的催化结构域(α-domain，residues 311 to 629)进行定向进化，以类胡萝卜素合成途径作为筛选标记[35]，通过菌落的颜色定向筛选出突变体，经过两轮筛选最终从突变体中筛选出高催化活性的蒎烯合成酶($PS_{mut}^{H346Y\text{-}Q457L}$)，之后组合代谢工程强化 MEV 途径的通量，再将蒎烯合成酶突变体($PS_{mut}^{H346Y\text{-}Q457L}$)和香叶基焦磷酸合成酶(*Abies grandis*，AgGPPS)融合表达，蒎烯终产量为 150 mg/L，比 PSwt (20 mg/L)高 6 倍多[36]。表 4 再简单介绍其他萜类合成酶定向进化的实例。

表 3. 不同植物来源的单萜环化酶/合成酶的自身序列与保守序列之间的比对[34]

Table 3. Native vs consensus sequences of the targeted enzymes from different plant mTC/S[34]

Targeted enzymes	Region 1	Region 2	Region 3
Consensus	*IALIT*	*IGGPVI*	*ARMAQFMY*
LimS	*NALIT*	*ISGPCM*	*GRMAQLMY*
PinS	*CHIIT*	*SGHRVS*	*SRAFHCGY*
FenS	*IALTT*	*ITCPTI*	*GRVANLAY*

LimS: limonene synthase from *Mentha spicata*; PinS: α-pinene synthase from *Pinus taeda*; FenS: fenchol synthase from *Lavandula viridis*. Each residue targeted by mutagenesisis marked in underline.

表 4. 萜类合成酶的定向进化的实例

Table 4. Examples of directed evolution of terpenoid synthases

Names	Strategies	Sites	Applications	Results or titer/(mg/L)	References
Geraniol	Site-directed mutation	*CrGES*$^{Y436A\text{-}D501A}$	The H-bonds between Asp/Tyr and the phosphate groups not only play an important role to geraniol formation, but also provide important clues for other monterpene synthases characterization and further optimization in a more rational way	The mutations of *CrGES*$^{Y436A\text{-}D501A}$ significantly decreased the affinity of GPP and geraniol synthase, consequently reducing geraniol production than the wild-type	[37]
Lycopene	Site-directed mutation	*CrtE*^{C81T}, *CrtYB11M*$^{W61R,\ S210S,G1221A}$	To obtain solely phytoene synthase function and further increase the FPP competitiveness of the lycopene synthesis pathway, enhancing the catalytic performance of *CrtE* and *CrtYB11M* by directed evolution	1610	[38]
Trichodiene	Site-directed mutation	TDS$^{N225D\text{-}S229T\text{-}N225D/S229T\text{-}Y295F}$	Exploring different TDS cyclization products by directed evolution	The content of terpenoids that contain β-farnesene, bisabolene, cuprenene, β-bisabolene, trichodiene, which has significantly differences from wild-type and mutant trichodiene synthases	[39]
S-Limonene	Site-directed mutation	LS$^{N345A/L423A/S454A\ or\ N345I}$	Revealing the plasticity of the active site and putting forward S-limonene synthetase (N345) of the polar amino acid sites is very important to the synthesis of limonene	S-limonene synthase can transform limonene into pinene or phellandrene by directed evolution	[40]

4 萜类合成酶定向进化的新策略

截止 2018 年 4 月，已经有 108 多个萜类合成酶的晶体结构发表(Protein Data Bank，http://www.rcsb.org)，其中包括 ClassⅠ和 ClassⅡ以及二者的组合体(ClassⅠ+Ⅱ)。这些萜类合成酶晶体结构的解析有助于人们能更全面、更系统地分析酶结构与功能的关联性，为进一步阐明酶的催化机制提供理论依据，并且也能从进化的角度了解萜类合成酶的进化历史，为挖掘更多的萜类合成酶提供参考。

针对未被解析的萜类合成酶，可利用多重序列比对软件 Clustal X、在线软件 ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi)与已知晶体结构的萜类合成酶的蛋白序列进行比对找出保守区，并结合已有的萜类合成酶的结构确定催化结构域的位置。与此同时可使用在线软件 SWISS-MODEL (https://swissmodel.expasy.org/)、Phyre 2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index)预测萜类合成酶的三维结构，预测好的三维结构可以使用软件 PyMol(https://pymol.org/2/#products)打开，并与相应已解析的萜类合成酶比对，再结合蛋白序列比对结果，采用同源建模的方式进一步找出与底物相互作用密切的位点及酶的催化活性口袋。

筛选是限制萜类合成酶定向进化的关键步骤，为了减少筛选的工作量同时兼顾突变文库的质量，可采取非理性和理性设计结合的半理性设计的方法，该法是一种目前应用非常广泛的酶的定向进化技术[41–42]。该法主要借助生物信息学将萜类合成酶的序列或结构等已有的信息和酶的定向进化进行组合，再借助计算机模拟手段，在酶催化口袋周围选取与底物直接相互作用的氨基酸残基，根据酶催化口袋的理化性质(如极性、非极性、结构类似、空间位阻大小不同的氨基酸等)，有针对地选取多个氨基酸作为改造靶点(2–4 个氨基酸可分为一组)，并理性设计某一特定的氨基酸密码子作为建构单元(如 NNK, NDT[4])，对催化口袋附近的氨基酸进行饱和突变(如 TCSM)[4]，有针对性地对萜类合成酶进行改造(如催化效率、底物专一性、立体选择性、稳定性)，重塑萜类合成酶的催化口袋。通过构建“小而精”高质量的突变文库，以 NNK 简并密码子(编码 20 种氨基酸)为例，设定95%文库覆盖度，筛选规模约为10^{15}，而 TCSM 筛选量降至200–800[32]，利用平板、高通量流式细胞仪荧光筛选等方法，从突变文库中筛选出高活性的萜类合成酶(图 3)。

5 讨论与展望

萜类化合物是数量最多的一类植物天然产物，在医药、食品、化工等领域应用广泛，具有非常广阔的开发及应用前景。近年来，国内外科研工作者对萜类合成酶的结构及功能方面的研究取得了很大的进展，越来越多的萜类合成酶的晶体被解析，这些研究成果对了解及阐明萜类化合物合成机理至关重要，为提高萜类合成酶的酶活提供了理论依据，也为开发更多具有市场价值的萜类化合物奠定了基础。萜类合成酶性能的好坏是萜类化合物异源合成的关键，但天然的萜类合成酶可能存在缺陷，不能满足人们的需求，其应用潜力也远远没有被挖掘。酶的定向进化可以有针对性地改造酶的性能，因此酶的定向进化技术将会成为改造酶的主流技术，但其仍然面临诸多挑战，其中筛选是制约酶定向进化改造的瓶颈[4]。如何有效地结合三种酶的定向进化策略，实现优势互补，构建高质量的多样性突变文库和高效、快速的筛选方法[27,43]，将会成为今后努力的方向。

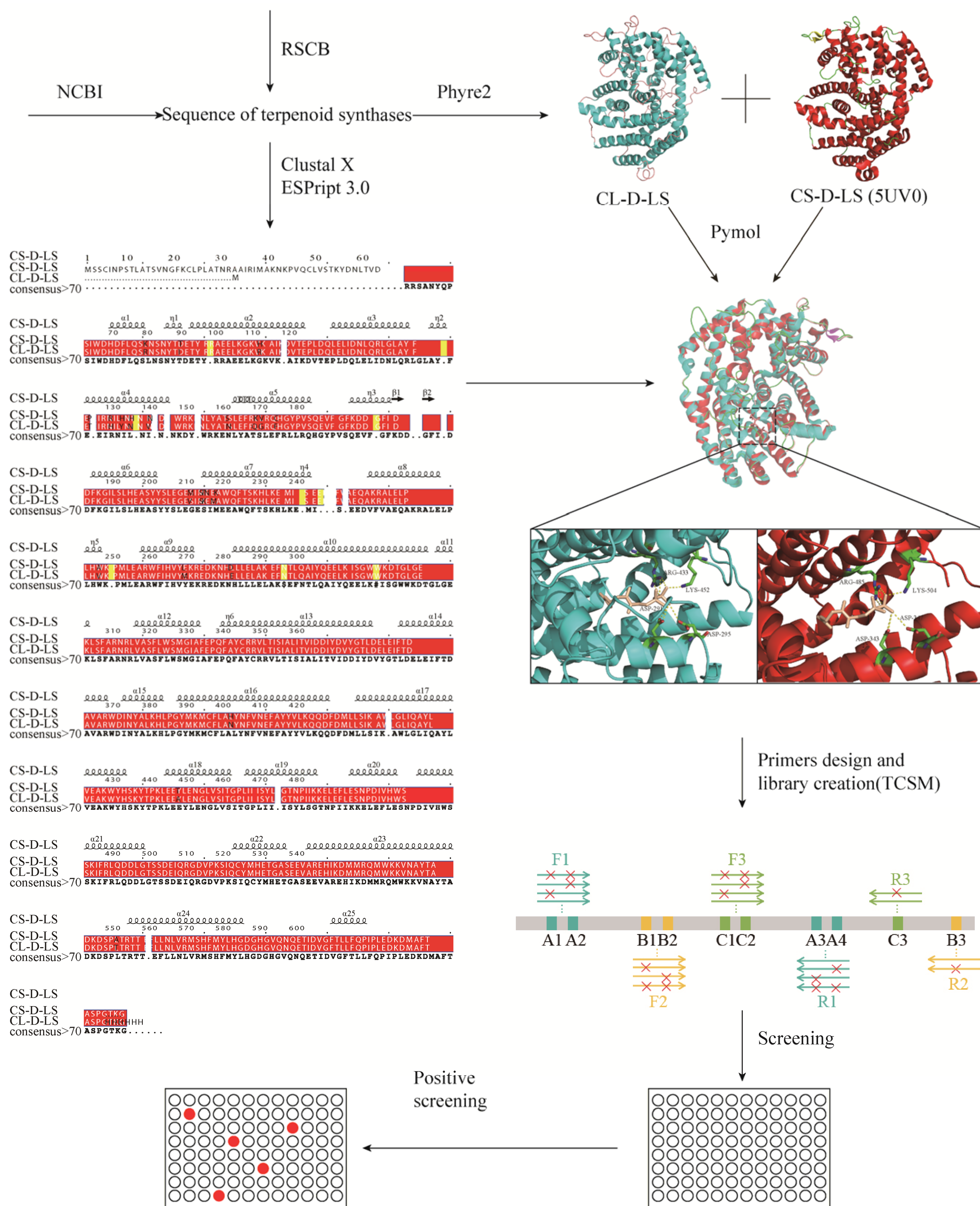

图 3. 萜类合成酶同源建模、突变文库构建及筛选的流程图

Figure 3. A flow diagram of the homologous modeling, creation and screening of mutant libraries of terpenoid synthases.

随着计算机模拟技术的发展，未来酶的定向进化走向基于计算机模拟的理性设计是必然趋势，但任重而道远。同时随着基因合成成本的降低，突变文库全基因合成不仅提高了文库的构建速度和文库序列的多样性，而且还可以有效减少密码子引入的偏好性，因此该方法也将成为今后酶定向进化技术重要的发展方向。

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Innovations for directed evolution of terpenoid synthases

Zhihui Hu, Yanru Weng, Bingxu Chen, Aiqun Yu, Dongguang Xiao*

Key Laboratory of Industrial Fermentation Microbiology, Ministry of Education, College of Biotechnology, Tianjin University of Science & Technology, Tianjin Engineering Research Center of Microbial Metabolism and Fermentation Process Control, Tianjin 300457, China

Abstract: Terpenoids are mostly existing compounds in natural products like plants and microorganisms. The application prospect of terpenoids attracts much attention owing to more and more valuable terpenoids discovered. However, limited yield and high extraction cost of terpenoids restrict their wide applications. The rise of synthetic biology has provided new ideas for biosynthesis of valuable terpenoids using targeted and high efficient microbial cell factories. Although terpenoid synthases are widely used as target enzymes in metabolic regulation of terpenoids biosynthesis, many natural terpenoid synthases have some disadvantages, such as insufficient catalytic activity, poor substrate specificity, poor regio- or stereoselectivity, poor stability and so on, which unfavorably affect the yield of terpenoids. To solve above problems, directed evolution of terpenoid synthases has been applied, which will have profound impact on biosynthesis of terpenoids by microbial cell factories. This review summarizes recent advances and their applications in directed evolution of enzymes. Meanwhile, the strategies for directed evolution of terpenoid synthases are proposed.

Keywords: terpenoids, terpenoid synthases, synthetic biology, directed evolution

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*Corresponding author. Tel: +86-22-60600019; E-mail: xiao99@tust.edu.cn

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